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Low-temperature storage of cucumbers induces changes in the organic acid content and in citrate synthase activity

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ABSTRACT

To elucidate the cause of reported pyruvate accumulation in chilled stored cucumbers (*Cucumis sativus* L.) cv. 'Toppugurin', we have examined differences in the extent of incorporation of acetate-1,2-^{14}C into the tricarboxylic acid (TCA) cycle and the specific activity of the enzyme citrate synthase between healthy and chilling-injured cucumber tissues stored in the dark for 0, 3, 6 and 12 d at 1 °C or 20 °C. Radioactive tracing, gas chromatography, and enzyme analysis suggest that the incorporation of acetate into citrate, the distribution of organic acids in the tricarboxylic acid cycle, and the specific activity of citrate synthase in the cold-storage (chilling-injured) cucumber tissues differed from those in the healthy tissues. The observed decrease in citrate synthase activity may be the cause of the observed increase in pyruvate accumulation in the chilling-injured cucumber fruit tissue compared to tissues stored at 20 °C. The results also suggest that the increased formation of pyruvate and reduced activity of citrate synthase during cold storage observed in the present study could serve as indicators (biomarkers) of stress-induced changes in chilled cucumbers. Possible mechanisms of the described effects are discussed.

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1. Introduction

The cucumber (cucurbits) is an important fruit vegetable belonging to the family *Cucurbitaceae*. Cucumbers contain approximately 95% water, 3.6% carbohydrates, and 0.65% protein, and are low in calories (150 kcal kg^{-1}). They are a good source of the following nutrients (in mg kg^{-1}): pantothenic acid (B_5) (0.026); vitamin C (0.28); magnesium (1.3) (USDA, 2008). Cucumbers are grown both as a source of pickles, and to be eaten fresh. While consumption of pickles has been waning, the use of cucumbers as a fresh vegetable has been increasing (Lucier and Jerardo, 2007).

To avoid deterioration, cucumbers are often stored and transported at cold temperatures (chilled). However, cucumbers are among a number of tropical and subtropical crops that are sensitive to above-freezing cool temperatures. Low-temperature storage of cucumbers may induce damage associated with chilling injury (CI), resulting in compositional as well as in morphological (structural) and microbiological changes (Fernández-Trujillo and Martínez, 2006; Fukushima et al., 1977; Marcellin and Ulrich, 1983; Martínez and Fernández-Trujillo, 2007; Musmade and Desai, 1998; Pérez-Díaz and McFeeters, 2008; Tatsumi et al., 1987, 2006; Wang, 1989). Not all cucumber cultivars are equally sensitive to chilling, but one study found that all tested varieties showed some injury during storage at 1 °C (Hakim et al., 1999).

Tissue injury in response to cold appears to be induced by sudden cessation of cellular cytoplasmic streaming and a change in membrane fluidity at threshold temperatures, impairing the cells ability to transport critical substrates, metabolites, and control molecules throughout the cell. This, in turn, leads to a metabolic imbalance and accumulation of toxic products (Brecht et al., 2008; Lyons and Breidenbach, 1987; Sevillano et al., 2009). CI-resistant plants are able to overcome cold temperatures by lowering cellular susceptibility, e.g. incorporation of a higher percentage of unsaturated lipids in the cellular membrane (Nishida and Murata, 1996; Orr and Raison, 1990), and by increasing effectiveness of defense and repair mechanisms against reactive oxygen species, e.g. increased superoxide dismutase and catalase activities (Wang, 1995).

Previous studies showed that concentrations of alanine increased in CI-sensitive crops during cold storage (Kozukue et al., 1984; Patterson et al., 1981). Subsequently, it was shown that conversion of pyruvate to alanine by glutamate-pyruvate transaminase (GPT) was likely the source of the increased alanine, as levels of pyruvate were found to be very high in chilled cucumbers and egg-

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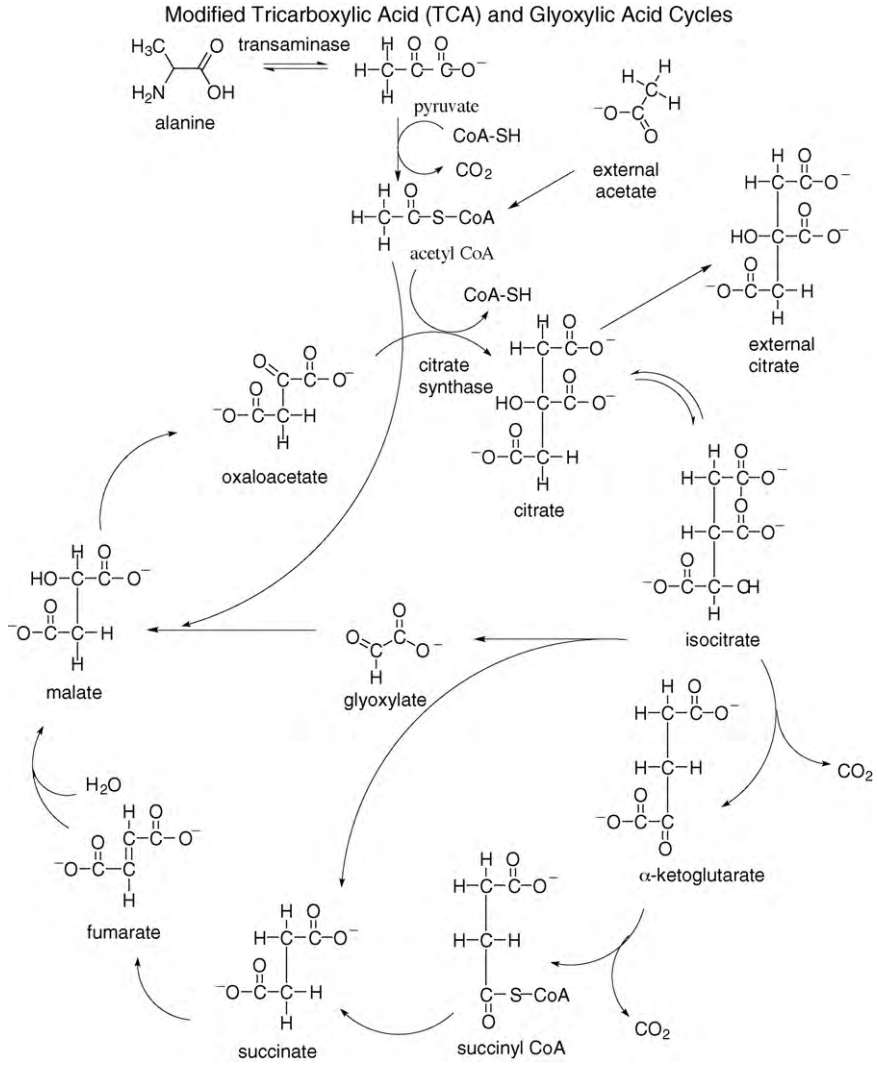

Fig. 1. Incorporation of acetate into tricarboxylic and glyoxylate cycles in plant tissues.

plants while GPT activity was unaffected by the chilling (Tsuchida et al., 1990). Other investigators have also found elevated pyruvate levels in chilled cucumbers (Hakim et al., 1999). Pyruvate is centrally important in the tricarboxylic acid (TCA) cycle (Fig. 1) for aerobic respiration. Respiration during chilling is altered in both CI-sensitive and CI-insensitive crops (Atkin and Tjoelker, 2003). For example, Murata (1969) found that the TCA pathway from oxaloacetic to citric acid in a banana peel was inhibited by cold storage. It was also reported that the α-keto acids oxoglutaric and oxaloacetic, intermediates in the TCA cycle, rapidly accumulated in the tissues of cucumbers (Hirose, 1987). The accumulation of pyruvate in CI-sensitive crops may be caused by a dysfunction of the pyruvate dehydrogenase complex, which participates in the formation of acetyl CoA from pyruvate or by a dysfunction of the enzyme citrate synthase, which participates in the formation of citrate from oxaloacetate and acetyl CoA (Fig. 1). To our knowledge, no studies have been published on the cause of pyruvate accumulation in CI-sensitive crops during cold storage.

The main objective of the present study was to determine the difference between chilled and non-chilled cucumbers in the incorporation of acetate-1,2-^{14}C into the tricarboxylic acid (TCA) cycle as a measure of pyruvate utilization, and whether this difference is reflected in the activity of citrate synthase in healthy and chilled cucumber tissues.

2. Materials and methods

2.1. Materials

Mature cucumbers (*Cucumis sativus* L.) cv. 'Toppugurin' of unknown history were purchased at a local market in Kobe, Japan and immediately stored in the dark at 1 °C or 20 °C for 0, 3, 6 or 12 d (Hakim et al., 1999). Sodium acetate-1,2-^{14}C (50 Ci mol^{-1}, ICN-Biochemicals, purity > 99%) was purchased from Japan Isotopic Corporation, Tokyo, Japan. Acetyl coenzyme A and acetylpyridine-adenine dinucleotide were obtained from Sigma (St. Louis, MO), malate dehydrogenase from Oriental Yeast Co. (Tokyo, Japan), and L-malate and triethanolamine HCl buffer from Wako Pure Chemicals (Osaka, Japan).

2.2. Incorporation of acetate-1,2-^{14}C into cucumber tissues

Incorporation experiments with radioactive acetate were performed in tightly closed glass tubes (50 mL; 3 cm i.d. × 15 cm) by incubating sliced tissues (5 g; 2–3-cm thick) from the center part of the cucumber fruit in 1.15 M phosphate buffer (20 mL; pH 7.0, matching the pH of the fruit) containing 2 μCi of uniformly labeled acetate 1,2-^{14}C. A small vial containing 2N KOH (2 mL) was placed into the test tube to trap CO_2 produced by the sliced tissues,

as described in related studies (Kozukue and Friedman, 2003; Kozukue et al., 2001). The test tubes were incubated at 15 °C for 2 h and 4 h with gentle shaking. After incubation, the solution was suspended in a toluene scintillator for the determination of radioactive CO_2 produced by the tissues. The incubated tissues were rinsed thoroughly with distilled water, frozen in liquid nitrogen, and stored at −40 °C for the following experiments.

2.3. Extraction of organic acids from cucumber tissues

The frozen tissues (5 g) were transferred to a 100 mL Erlenmeyer flask fitted with a reflux condenser. To the flask was added hot aqueous/methanol (20/80, v/v; 50 mL), a solvent we previously used to extract amino acids and sugars from bamboo shoots (Kozukue et al., 1983). The mixture was then boiled for 5 min. After homogenization of the cooled sample in a Bio-mixer (Nihonseiki, Kobe, Japan), the samples were centrifuged at 9000 × *g* for 10 min. The pellet was re-extracted twice with hot 80% methanol/water and then centrifuged. The pellet was then dried at room temperature and the radioactivity counted in the toluene scintillator. The soluble extracts were combined and evaporated to dryness at 35 °C under reduced pressure, and then re-dissolved in the hot 80% methanol.

The methanol-soluble fraction was percolated though a column of DOWEX 50W-X8 (H^+) cationic resin. The basic and amphoteric fractions that were adsorbed on the cationic resin were eluted with 2N ammonia–water. The non-adsorbed neutral fraction on the cationic resin was percolated though a column of DOWEX 1-4X ($HCOO^-$) anionic resin. The acidic fraction adsorbed on the anionic resin was eluted with 6N formic acid. The individual fractions were evaporated to dryness at 35 °C and re-dissolved in 80% methanol (5 mL). An aliquot (1 mL) of this solution was suspended in the toluene scintillator for the determination of the radioactivity of individual fractions.

2.4. Thin-layer chromatography (TLC) of cucumber organic acids

The organic acid fraction was separated on TLC polyamide layer sheets (Chen Chin Trade Co., Kobe, Japan). The developing solvent was isopropyl ether:formic acid:distilled water (90:7:3, v/v/v). The spraying reagent was a solution of 0.1% Bromophenol Blue (BPB) in ethanol. Authentic citrate, malate, succinate and fumarate standards were used to identify the acids. Bands corresponding to these acids on the polyamide sheets were separately collected and suspended in the toluene scintillator for determination of individual radioactivity.

2.5. Gas–liquid chromatography (GC) of cucumber organic acids

The method was adapted from previous studies (Kozukue et al., 1978, 1983, 1984). Conditions: gas chromatograph, Shimazu model GC-9A 9T (Shimazu Co., Tokyo, Japan) with a hydrogen flame ionization detector; column, silicon OV-1 capillary (Shimazu HiCap, CBPI, 25 m × 0.5 mm); initial column-oven temperature, 100 °C programmed linearly to increase 0.033 °C s^{-1} to 270 °C; injection port and detector temperatures, 290 °C; flow rate of N_2 carrier gas, 0.833 mL s^{-1}.

Aliquots (1 mL) of the organic acid fractions were transferred to 3 mL vials, lyophilized to dryness, and further dried under reduced pressure over P_2O_5. The organic acids were then converted to trimethylsilyl (TMS) derivatives with *bis*-trimethylsilylacetamide (100 μL):acetonitrile (1:4, v/v) for 60 min at room temperature. Aliquots (2 μL) of the derivatives were injected into the gas chromatograph equipped with an integrator (Shimazu model 2B).

2.6. Determination of radioactivity of cucumber extracts

The following substances were each suspended in a toluene scintillator containing PRO (2,5-diphenyloxazole) (0.6 g) and POPOP (1,4-*bis*-2-phenyloxazolebenzene) (0.5 g) in toluene (667 mL) plus Triton X-100 (333 mL): trapped CO_2 produced during incubation of tissues; 80% methanol-soluble and insoluble fractions; three ion exchange fractions from the 80% methanol-soluble fraction; and organic acids separated by TLC. The radioactivity of the suspensions was then determined with the aid of the Aloka Liquid Scintillation System (LSC-3500).

2.7. Preparation of citrate synthase from cucumber tissues

The enzyme was isolated by a modification of the procedure described by Mukherjee and Srere (1976). Frozen cucumber tissues (5 g) were placed in a mortar, chilled with liquid nitrogen, and pulverized to a fine powder. The powders were then homogenized with cold potassium phosphate buffer (5 mM, pH 7.4) containing phenylmethylsulfonyl fluoride (PMSF, 300 μM) and 2-mercaptoethanol (2-ME, 1.4 mM). The homogenate was filtered though three layers of cheesecloth and centrifuged at 9000 × *g* for 10 min to remove debris. The resultant supernatant was brought to 75% saturation with ammonium sulfate. The pellet was collected by centrifugation (9000 × *g*, 5 min, 5 °C), suspended in potassium phosphate buffer (5 mM) containing PMSF (300 μM) and dialyzed overnight at 5 °C against the solvent. The resultant dialyzed solution was used for protein determinations and enzyme assays.

2.8. Citrate synthase assay of cucumber extracts

Citrate synthase activity was measured by following the rate of 3-acetylpyridine-adenine dinucleotide reduction at 365 nm in a spectrophotometer (Stitt, 1984). The dialyzed enzyme solution (130 μL) was incubated with triethylamine–hydrochloric acid buffer, pH 8.5 (916 μL, 0.7 M), potassium malate, pH 7.5 (26 μL, 140 mM), acetylpyridine-adenine dinucleotide (26 μL, 10 mM), and malate dehydrogenase (26 μL, 600 kU L^{-1}, pH 8.6) at 25 °C for 5 min. Next, acetyl coenzyme A solution (26 μL, 8.4 mmol L^{-1}) was added and the increase in absorbance per unit of time was measured. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard. The specific activity of citrate synthase was expressed as units per kilogram of protein, U kg^{-1}.

3. Results

Table 1 shows the specific radioactivity of CO_2, and methanol-soluble and insoluble fractions from stored cucumber tissues incubated with acetate-1,2-^{14}C for 2 h and 4 h. ANOVA tests were performed on the results with the aid of SigmaPlot 11 (Germany). Samples were significantly different with $P < 0.005$. Evolved radioactive CO_2 is a measure of acetate used by the fruit for respiration. Radioactivity in the methanol-soluble and insoluble fractions measure the acetate used for incorporation into the tissues. Together, these three measures give an estimate of total acetate utilization of the fruit. Although it was previously reported that acetate-1,2-^{14}C was utilized by young plant tissues within 2 h (Walsh and Koshland, Jr., 1984), the specific radioactivity of CO_2 produced by cucumber tissue incubated with acetate-1,2-^{14}C for 4 h was higher than that in the cucumber tissues incubated for 2 h ($P = 0.045$). Therefore, 4-h incubations were used for the remaining experiments.

As seen in Table 1, there were considerable differences in the utilization of acetate-1,2-^{14}C between the tissues stored at 20 °C and at 1 °C ($P < 0.001$). The rates at 20 °C were higher. For both

Table 1
Specific radioactivities of three fractions obtained from fresh cucumber fruit tissues incubated with acetate-1,2-^{14}C.

Storage temperature[a]	Days in storage[b]	Incubation time (h)[c]	Specific radioactivity, kBq kg^{-1}			
			CO_2	Methanol-soluble	Methanol-insoluble	Total
	Initial	2	3.4 ± 0.3 (0.6)[*,1]	444.2 ± 29.2 (72.1)[1,2,3]	168.8 ± 113.1 (27.4)[1,2]	616.5[1]
		4	4.1 ± 0.2 (0.4)[1]	615.7 ± 36.8 (62.8)[1,2]	360.0 ± 24.4 (36.7)[3]	979.8
1 °C	3	2	117.9 ± 10.2 (8.0)[2]	1221.8 ± 58.7 (83.2)[4,5]	129.7 ± 9.2 (8.8)[1,2]	1469.3[2]
		4	150.6 ± 10.5 (12.5)[3]	846.3 ± 53.5 (70.1)	210.1 ± 16.6 (17.4)[1]	1207.1[3]
	6	2	38.3 ± 2.5 (5.5)[3]	547.4 ± 35.2 (78.9)[2,3]	108.0 ± 7.0 (15.6)[1,2]	693.6[1]
		4	69.5 ± 5.2 (12.3)[4]	377.2 ± 20.7 (66.7)[3,6]	119.2 ± 7.0 (21.1)[1,2]	565.9[1]
	12	2	29.0 ± 1.8 (9.1)[3]	244.4 ± 17.0 (76.9)[6]	44.6 ± 3.3 (14.0)[2]	318.0[4]
		4	37.7 ± 3.1 (12.3)[3]	222.0 ± 13.7 (72.5)[6]	46.6 ± 2.5 (15.2)[2]	306.4[4]
20 °C	3	2	104.1 ± 9.0 (4.0)[2]	2319.2 ± 150.0 (88.9)	185.7 ± 13.1 (7.1)[1]	2609.0
		4	194.1 ± 15.4 (10.0)	1569.6 ± 118.5 (81.0)	173.2 ± 13.3 (8.9)[1,2]	1936.9
	6	2	33.4 ± 2.1 (2.3)[3]	1290.0 ± 81.6 (87.3)[4]	154.8 ± 13.5 (10.5)[1,2]	1478.2[2]
		4	56.5 ± 3.7 (4.3)[4]	1038.1 ± 65.0 (78.4)[7]	229.7 ± 18.7 (17.3)[1,3]	1324.2[2,3]
	12	2	27.0 ± 2.5 (1.8)[3]	1315.8 ± 64.3 (89.3)[4]	130.2 ± 10.4 (8.8)[1,2]	1473.0[2]
		4	38.0 ± 3.1 (3.0)[3]	1106.7 ± 66.5 (87.1)[5,7]	126.1 ± 10.8 (9.9)[1,2]	1270.9[2,3]

[1–7] No statistical difference by ANOVA within columns.
[a] 1 °C treatment significantly different than 20 °C treatment.
[b] 3 d of storage significantly different than all other storage days.
[c] 2 h not significantly different than 4 h.
[*] Listed values are averages ± SD (n = 3). Values in parenthesis are % of total radioactivity.

storage temperatures, acetate-1,2-^{14}C incorporation was highest in the cucumber stored for 3 d (P < 0.001). Thereafter, the incorporation of acetate into tissues markedly decreased with further storage (P = 0.001), especially for tissues stored at 1 °C.

To obtain additional details about the distribution of radioactivity, the methanol-soluble extracts were further fractionated by ion exchange chromatography into acidic, basic-amphoteric, and neutral fractions. Table 2 shows specific radioactivity of the acidic, basic plus amphoteric, and neutral fractions obtained after passing the methanol-soluble fraction through two ion exchange resins. The acidic fraction contained most of the radioactivity in the methanol-soluble fraction, as much as 80% higher for the 20 °C, 3 d sample. The radioactivity in the acidic fraction peaked for the sample stored 3 d at both storage temperatures. The activity at 20 °C was approximately double that at 1 °C. All stored cucumbers showed a decrease in the radioactivity of the neutral fraction (P < 0.001). The basic and amphoteric fractions showed a gradual decrease over time in radioactivity at 1 °C storage, and a slight increase at 20 °C at 3 d. It then leveled off at 6 and 12 d. Overall, the ratios of acidic to basic fractions ranged from about 1.2 to 4.0 and the ratio of the acidic to neutral fractions, from about 7 to 120.

Specific radioactivity of the major organic acids isolated by GC and TLC from the 80% methanol-soluble acidic fraction are listed in Table 3. Overall, the ratios of acidic to basic fractions ranged from about 1.2 to 4.0 and the ratio of the acidic to neutral fractions, from about 7 to 120. The synthesis of the more abundant organic acids, malic and citric, increased dramatically in cucumbers stored for 3 d (3.3–3.5X at 1 °C; 6.8–7.0X at 20 °C) and decreased in cucumbers stored longer. However, whereas synthesis of both organic acids remained high in cucumbers stored at 20 °C for up to 12 d (~4X initial values), the synthesis in cucumbers stored at 1 °C for 6 or 12 d returned to (malic acid) or decreased from (citric acid) initial rates. The ratio of citric to malic acid synthesis remained relatively unchanged except for the sample stored at 1 °C for 12 d, where citric acid synthesis decreased to about one-fifth that of the initial rate.

Trends in the specific activities of citrate synthase in the cucumber tissues stored at 1 °C and 20 °C are depicted in Fig. 2. Compared to initial activity, specific activity of citrate synthase in the tissue stored at 20 °C did not change, even after storage for 12 d. However, the enzyme activity in the tissues stored at 1 °C decreased by about 20% after 3 d, and by about 30% after 12 d.

4. Discussion

Pyruvate and acetate are both precursors for the synthesis of citrate in the TCA cycle. Tracking the uptake of acetate can be a useful indicator of pyruvate utilization as well as a measure of cellular respiration. In plant cells, acetate is converted to acetyl CoA, which is utilized in two metabolic pathways: the tricarboxylate and glyoxylate cycles (Berry, 1971; Maclenan et al., 1963; Schwimmer, 1981; Walsh and Koshland, Jr., 1984). We did not detect any glyoxylate

Table 2
Specific radioactivities of three fractions prepared from 80% methanol/water-soluble fraction of stored fresh cucumber fruit tissues treated with acetate-1,2-^{14}C for 4 h and after storage for three time periods at 1 °C and 20 °C.

Storage temperature	Days in storage	Specific radioactivity, kBq kg^{-1}		
		Acidic	Basic and amphoteric	Neutral
	0	322.12 ± 26.08 (52.31)[*,1]	250.03 ± 16.42 (40.61)[1,2]	43.58 ± 3.57 (7.08)
1 °C	3	612.55 ± 42.35 (72.38)	228.67 ± 14.92 (27.02)[1]	5.12 ± 0.30 (0.60)[1]
	6	230.50 ± 16.83 (61.10)[1,2]	132.50 ± 14.92 (35.12)	14.23 ± 1.10 (3.77)[2]
	12	148.13 ± 7.75 (66.72)[2]	65.82 ± 4.75 (29.64)	8.08 ± 0.92 (3.64)[1]
20 °C	3	1248.13 ± 85.03 (79.52)	307.37 ± 22.52 (19.58)[3]	14.10 ± 1.28 (0.90)[2]
	6	745.37 ± 22.02 (71.80)[3]	276.38 ± 18.37 (26.62)[2,3]	16.32 ± 1.32 (1.57)[2,3]
	12	803.63 ± 41.68 (72.61)[3]	283.65 ± 17.08 (25.63)[2,3]	19.43 ± 1.68 (1.76)[3]

[1–7] No statistical difference by ANOVA within columns.
[*] Listed values are averages ± SD (n = 3). Values in parenthesis are % of total radioactivity.

Table 3
Specific radioactivity of organic acids isolated from the 80% methanol/water-soluble acidic fraction of stored fresh cucumber tissues treated with acetate-1,2-^{14}C for 4 h.

Storage temperature	Days in storage	Specific radioactivity, kBq kg^{-1}				
		Succinic	Fumaric	Malic	Citric	Citric/malic
1 °C	0	30.37 ± 3.33	10.68 ± 0.97[1]	71.13 ± 8.50[1]	93.48 ± 8.80[1]	1.31
	3	17.38 ± 3.13[1,2]	4.72 ± 0.47[2]	249.40 ± 24.35[2]	308.73 ± 2.60	1.24
	6	19.63 ± 1.63[1]	5.33 ± 0.45[2]	86.88 ± 7.63[1]	93.00 ± 5.75[1]	1.07
	12	12.27 ± 1.13[2]	10.83 ± 0.87[1]	71.98 ± 6.68[1]	17.53 ± 1.92	0.24
20 °C	3	62.58 ± 3.58	13.15 ± 1.13[1]	501.12 ± 25.55	633.67 ± 22.13	1.26
	6	41.28 ± 2.50	11.13 ± 0.77[1]	276.42 ± 20.20[2,3]	372.25 ± 25.30[2]	1.35
	12	50.32 ± 3.35	17.30 ± 1.62	317.37 ± 15.30[3]	359.57 ± 17.02[2]	1.13

Listed values are averages ± SD (n = 3). [1–7] No statistical difference by ANOVA.

in cucumber tissues by capillary gas liquid chromatography (Fig. 3) or by TLC.

The observed decreases in citrate synthase activity and in respiration during low-temperature storage may explain the previous observation that pyruvate accumulates in CI cucumbers. A related study reported that the activity of citrate synthase in banana peel was inhibited in the initial stage of CI (Abd El-Wahab and Nawwar, 1977). It is possible that the same factors that are causing a decrease in citrate synthase activity also cause a decrease in pyruvate dehydrogenase activity. NADH accumulation is known to inhibit both enzymes. Because pyruvate formation is normally inhibited by excess citrate, the observed low formation in chilled cucumbers may further increase the levels of pyruvate.

It is well known that most of the structural and functional properties of allosteric enzymes are temperature sensitive (Schwimmer, 1981). These enzymes regulate key reactions involved in metabolic pathways. Most allosteric enzymes are reversibly inhibited by cold temperatures (Jaenicke, 1981; Schwimmer, 1981). Because citrate synthase is also an allosteric enzyme, the above described results suggest that citrate synthase activity may also be irreversibly inhibited by chilling temperatures.

Mechanisms of citrate synthase formation and inhibition as well as the catalysis of pyruvate formation and the properties of the pyruvate dehydrogenase complex at low temperatures remain to be elucidated. It would also be of interest to find out whether the enhanced pyruvate synthesis and reduction of citrate synthase activities in low-temperature stored cucumbers could serve as indicators (biomarkers) of the extent of damage caused by chilling and possibly other stress conditions in numerous other fruits and vegetables (Marcellin, 1992; Moline, 1987; Musmade and Desai, 1998; Wang, 1989).

The results of radioactivity measurements of acidic, basic, and neutral fractions (Table 2) suggest that relative contributions of

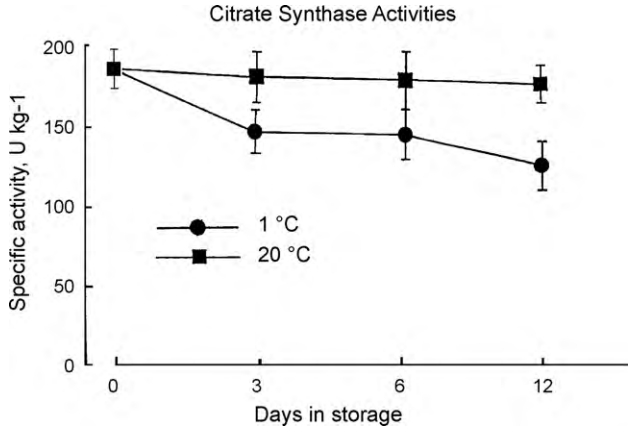

Fig. 2. Specific activities of citrate synthase of cucumbers stored at 20 °C and 1 °C. Bars indicate LSD at 0.05.

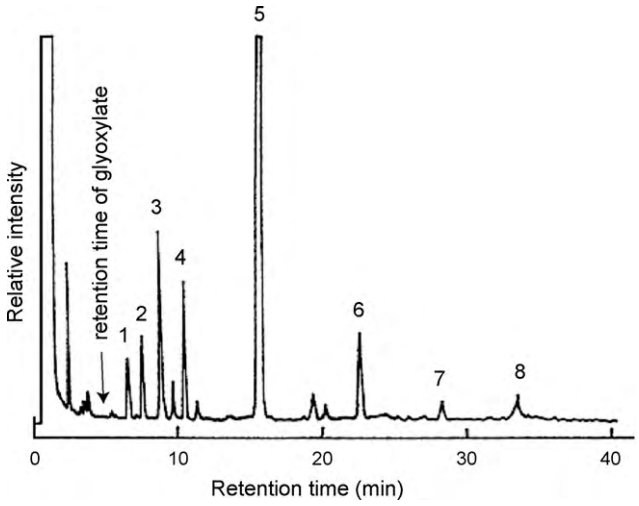

Fig. 3. Gas chromatogram of the acidic fraction of a fresh cucumber extract. Peaks: (1) unknown; (2) succinic acid; (3) fumaric acid; (4) unknown; (5) malic acid; (6) unknown, (7) unknown; and (8) citric acid.

the three fractions to the total radioactivity are influenced by both temperature and time of storage of the cucumbers. We did not determine the nature of the metabolites associated with each fraction. It is likely that the acidic fractions contain mostly organic acids, the neutral fractions, free amino acids, and the basic fractions, free sugars.

5. Conclusion

Using radiolabeling techniques, gas chromatography, and enzyme assays, we observed in the present study changes in the distribution of organic acids and reduction in the activity of citrate synthase in cucumber tissues stored at 1 °C for up to 12 d. The results of the present and of previous cited studies suggest that increased synthesis of alanine and pyruvic acid and reduced activity of citrate synthase could serve as biomarkers of stress-induced changes during storage of chilled cucumbers.

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